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Award Number: W81XWH-04-1-0079

TITLE: The Role of Mutant p53 in Progression of Prostate Cancer

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REPORT DATE: December 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 01-12-2005		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 Dec 2003 – 30 Nov 2005	
4. TITLE AND SUBTITLE  The Role of Mutant p53 in Progression of Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0079	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)  Gang Liu, M.D., Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  University of Alabama at Birmingham Birmingham, AL 35294				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT p53 mutation is frequently found in advanced prostate cancers (CaP), such as androgen independent CaP, which suggests that mutant p53 may have a role in the progression of CaP. By generating of a series of prostate cancer cell lines, in which wild-type or mutant p53 is knocked down and (or) various mutant p53 are simultaneously expressed, we found that knock-down of wild-type p53 confers CaP cells resistance to DNA damage-induced apoptosis. In addition, knock-down of mutant p53 renders CaP cells more sensitive to DNA damage-induced apoptosis. However, CaP cell death upon deprivation of androgen is independent of wild-type p53. Moreover, knock-down of mutant p53 is unable to convert the androgen-independent caP cells to androgen-dependent ones. Interestingly, re-introduction of mutant p53 into wild-type p53 knock-down CaP cells further increases their resistance to DNA-damage induced apoptosis. More importantly, re-introduction of mutant p53 into wild-type p53 knock-down CaP cells promotes their androgen-independent growth. Finally, we found that p53 activation upon DNA damage is impaired by deprivation of androgen.					
15. SUBJECT TERMS CaP; androgen-independent; mutant p53					
16. SECURITY CLASSIFICATION OF:			UU	18. NUMBER OF PAGES  22	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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## **Introduction**

p53 is one of the most important tumor suppressors in cells. Mutation of p53 has been found in more than 50% of human cancers (Kastan et al., 1991; Lane, 1992). In unstressed cells, p53 is maintained at a very low level by its negative regulators, such as Mdm2. Upon cellular stresses, p53 is activated and the activated p53 induces a series of target genes, including p21, Bax, and Puma, which mediate cell cycle arrest, apoptosis, senescence, and differentiation (Hansen and Oren, 1997a; Ko and Prives, 1996; Levine, 1997; Prives and Hall, 1999; Vogelstein et al., 2000). p53 mutation is not common in primary prostate cancers, but is frequently found in advanced CaP, such as metastatic CaP and androgen-independent CaP (Downing, 2001; Linderholm, 2001), which suggests mutant p53 may have a role in the progression of CaP.

## Main body

1. Knock-down of wild-type p53 confers prostate cancer (CaP) cells resistance to DNA damage-induced apoptosis.

To explore the role of p53 in the progression of CaP, we generated inducible p53 knock-down LNCaP cell line, in which p53 siRNA was inducibly expressed by the tetracycline-regulated H1 promoter. One representative clone, LNCaP-pTer-p53, was shown in Fig 1A. We performed growth curve assay and found that the growth of LNCaP cells showed no difference when p53 siRNA was inducibly expressed (data not shown). To investigate the role of p53 in regulation of DNA damage response, we performed cell survival assay. We found that more LNCaP cells survived DNA damage once p53 was knocked down (Fig. 1B). We also directly measured the dead cells after DNA damage by trypan blue dye exclusion assay and found that less LNCaP cells died when p53 was inducibly knocked down (Fig. 1C). To examine the long-term effect of p53 knock-down on CaP cell survival after DNA damage, we performed clonogenic assay. Significantly more LNCaP cells survived and formed colonies when p53 was inducibly knocked down (Fig. 1D). Thus, p53 has a critical role in apoptosis of LNCaP cells induced by DNA damage.

2. Knock-down of mutant p53 renders CaP cells more sensitive to DNA damage induced apoptosis.

To explore the role of mutant p53 in the response of CaP cells to DNA damage, we generated stable mutant p53 knock-down DU-145 cells. One representative clone, DU-145-p53KD, was shown in Fig. 2A. The proliferation of the parental cells, DU-145, and the p53 knock-down cells, DU-145-p53KD, was similar (data not shown). Next, we performed survival assay, trypan blue dye exclusion assay, and clonogenic assay. We found that less DU-145-p53KD cells survived DNA damage than the parental cells DU-145 did (Fig. 2B-2D).

3. CaP cell death upon deprivation of androgen is independent of wild-type p53.

The growth and differentiation of prostate tissue are dependent on androgen. This is also the case for CaP. CaP cells undergo apoptosis once they are deprived of androgen. However, CaP cells can eventually survive androgen deprivation and become androgen-independent. To explore the role of p53 in CaP cells' apoptosis induced by deprivation of androgen, we cultured LNCaP-pTer-p53 in the medium containing androgen-depleted serum. We found that LNCaP-pTer-p53 cells that are uninduced or induced to knock down p53 are equally sensitive to androgen depletion (Fig. 3).

4. Knock-down of mutant p53 is unable to convert the androgen-independent CaP cells to androgen-dependent ones.

Since we have found that LNCaP cell apoptosis induced by depletion of androgen is independent of wild-type p53. Moreover, p53 mutation is frequently found in advanced

CaP. Thus, if mutant p53 plays a role in the transition of CaP cells from androgen-dependency to androgen-independency, abrogation of mutant p53 in CaP cells could restore the androgen-dependency. To this end, we examined the androgen-dependence of DU-145-p53KD. We found that, similar to the parental cells (DU-145), DU-145-p53KD grows independent of androgen (data not shown). This suggests that p53 mutation is not the sole reason for androgen-independent transition, or the transition is irreversible.

5. Re-introduction of mutant p53 into wild-type p53 knock-down CaP cells further increases their resistance to DNA-damage induced apoptosis.

We have found that knock-down wild-type p53 has no effect on the androgen-dependent growth of LNCaP cells. We also found that knock-down of mutant p53 can not restore the androgen-dependence of DU-145 cells. However, we are considering that re-introduction of mutant p53 into wild-type p53 knock-down LNCaP cells may render these cells androgen-independent. Thus, we generated stable LNCaP cell lines, in which wild-type p53 was stably knocked down and one of p53 mutants was able to be inducibly expressed. These p53 mutations include: R175H that affects the conformation of p53 for binding to specific DNA sequence and R273H that disrupts the p53-DNA contacting site. These cell lines were designated as LNCaP-p53KD&p53(R175H) and LNCaP-p53KD&p53(R273H). One representative clone of LNCaP-p53KD&p53(R175H) was shown in Fig. 4A. We then performed cell survival assay and trypan blue dye exclusion assay and found that more LNCaP-p53KD&p53(R175H) cells survived the DNA damage than the parental LNCaP cells (Fig. 4B-4C). Moreover, when p53(R175H) was inducibly expressed, the LNCaP cells were even more resistant than LNCaP-p53KD to DNA damage-induced cell death (Fig. 4B-4C). Similarly, over-expression of p53(R273H) was able to significantly increase the resistance of LNCaP-p53KD cells to DNA damage-induced cell death (data not shown).

6. Re-introduction of mutant p53 into wild-type p53 knock-down CaP cells promotes their androgen-independent growth.

p53 mutation is not common in primary CaP, whereas p53 is frequently mutated in advanced CaP, including androgen-independent CaP. This suggests that mutant p53 alone has a role in CaP's transition from androgen-dependency to androgen-intendancy. To investigate whether mutant p53 is able to confer CaP cells the androgen-independent property, we examined the growth of various LNCaP cells cultured in androgen-depleted medium. We found that the wild-type p53 knock-down LNCaP (LNCaP-p53KD) cells is unable to proliferate in androgen-depleted medium. However, the LNCaP-p53KD was able to grow cultured in the same medium when p53(R175H) was inducibly expressed (Fig. 5). Similar to p53(R175H), p53(R273H) also promoted LNCaP-p53KD proliferation in androgen-depleted medium (data not shown). All of the evidence suggests that mutant p53 did have a role in androgen-independency of CaP.

7. p53 activation upon DNA damage is impaired by deprivation of androgen.

p53 is often regarded as the Guardian of genome. Inactivation of p53 leads to genomic instability, which could affect the functions of tumor suppressors and oncoproteins. Although we have found that apoptosis of LNCaP cells induced by deprivation of androgen is independent of p53, it can not be ruled out that p53 activation upon other cellular stresses is affected by deprivation of androgen. This could be a potential mechanism by which androgen-independent CaPs are resistant to apoptosis induced by androgen-deprivation. Thus, we determined the p53 activation by DNA damage before and after androgen deprivation. We found that p53 activation is significantly decreased upon DNA damage in LNCaP cells cultured in androgen-depletion medium compared with those in regular medium (Fig. 6, p53 panel). Moreover, p53 activation in colorectal cancer cell line RKO after DNA damage was not affected by androgen deprivation (data not shown). This suggests that defect in p53 activation caused by androgen deprivation is specific in CaP cells. CPT is type I topoisomerase inhibitor, which can generate DNA double-strand breaks. ATM is rapidly autophosphorylated and activated at serine 1981. Activated ATM is able to phosphorylate threonine 67 in Chk2 and serine 15 in p53, which will lead to p53 activation. Thus, we examined ATM autophosphorylation, Chk2 phosphorylation, and the phosphorylation of p53. We found that ATM and Chk2 phosphorylation were unaffected by androgen-deprivation (Fig. 6, p-S1981-ATM and p-T68-Chk2 panels). Phosphorylation of serine 15 in p53 was reduced by deprivation of androgen, which is likely to be the result of decrease in total p53 activation (Fig. 6, p-S15-p53 panel). Upregulation of p21, a p53 target, was impaired by deprivation of androgen (Fig. 6, p21 panel). However, the expression of Mdm2, the p53 negative regulator, was unchanged after DNA damage, although p53 level was significantly lower in cells cultured in androgen-deprivation medium than those in regular medium (Fig. 6, Mdm2 panel). All of the evidence suggests that androgen signaling pathway affects level of Mdm2 in CaP cells, which could be responsible for the defect in p53 activation after DNA damage in cells cultured with androgen-deprivation medium.

#### 8. Identification of novel target genes of mutant p53.

p53 mutation has been found in over 50% of human tumors. Mutant p53 loses wild-type p53 activities, such as cell cycle arrest and inducing apoptosis. In addition, mutant p53 is dominant negative over p53 family member proteins, including p53, p63, and p73. Furthermore, previous data showed mutant p53 has gain-of-function. However, the underlying mechanism remains largely elusive. To address this question, we generated a stable MIA-PaCa-1 cell line, in which the endogenous mutant p53 can be inducibly knocked down by siRNA. RNA was purified from MIA-PaCa-1 cells that are uninduced or induced to express p53 siRNA and a microarray assay was performed. We found that the expression of more than 20 novel genes has been altered after mutant p53 is knocked down. Most of these genes are involved in cell growth and death. We then performed Northern blot assay to confirm the microarray data. Fig. 7A is a representative blot. It was shown that the expression of epithelial protein up-regulated in carcinoma, membrane associated protein 17 (DD96), GRO1 oncogene (melanoma growth stimulating activity, alpha) (GRO1), and upregulated by 1,25-dihydroxyvitamin D-3 (VDUP1) is significantly decreased when mutant p53 is inducibly knocked down. Furthermore, we found that two putative oncogenes, ECT2 and  $\beta$ -catenin, are down-regulated when mutant p53 is

knocked down in MIA-PaCa-1 cells (Fig. 7B). These data suggest that mutant p53 confers cancer cells growth advantages by regulating down-stream targets, such tumor suppressors and oncogenes.

9. NINJ1, a novel p53 target gene, potentiates p53 activities in inducing cell cycle arrest and apoptosis. NINJ1 is an adhesive protein and involved in neurite outgrowth after axotomy. However, using DNA microarray, we found that NINJ1 is up-regulated by p53 and DNA damage, but not by p63 and p73 (Fig. 8A and 8B). To examine whether NINJ1 is a mediator of p53 activity, we generated stable MCF-7 cell line that can inducibly express NINJ1-HA. We found that over-expression of NINJ1-HA alone has no effect on cell growth, but potentiate p53-induced cell cycle arrest and apoptosis (Fig. 8C and 8D). To explore the mechanism underlying the potentiation effect, we examined the p53 transcriptional activity. We found that the p53 transcriptional activity is increased by over-expression of NINJ1-HA (Fig. 8E). However, the localization of NINJ1-HA remained unchanged by DNA damage. Thus the mechanism by which NINJ1 affect p53 activity needs further study (Fig. 8F).



## **Key research accomplishments**

1. Knock-down of wild-type p53 confers prostate cancer (CaP) cells resistance to DNA damage-induced apoptosis.
2. Knock-down of mutant p53 renders CaP cells more sensitive to DNA damage induced apoptosis.
3. CaP cell death upon deprivation of androgen is independent of wild-type p53.
4. Knock-down of mutant p53 is unable to convert the androgen-independent CaP cells to androgen-dependent ones.
5. Re-introduction of mutant p53 into wild-type p53 knock-down CaP cells further increases their resistance to DNA-damage induced apoptosis.
6. Re-introduction of mutant p53 into wild-type p53 knock-down CaP cells promotes their androgen-independent growth.
7. p53 activation upon DNA damage is impaired by deprivation of androgen.
8. Expression of epithelial protein up-regulated in carcinoma, membrane associated protein 17 (DD96), GRO1 oncogene (melanoma growth stimulating activity, alpha) (GRO1), and upregulated by 1,25-dihydroxyvitamin D-3 (VDUP1) is significantly decreased when mutant p53 is inducibly knocked down.
9. NINJ1, a novel p53 target gene, potentiates p53 activities in inducing cell cycle arrest and apoptosis.

## **Conclusions**

By generating a series of cell lines that can inducibly knock down endogenous wild-type p53 and (or) over-express various mutant p53, we found that CaP cell death upon deprivation of androgen is independent of wild-type p53. In addition, knock-down of mutant p53 is unable to convert the androgen-independent CaP cells to androgen-dependent ones. However, we found that re-introduction of mutant p53 into wild-type p53 knock-down CaP cells promotes their androgen-independent growth. Finally, we found that p53 activation upon DNA damage is impaired by deprivation of androgen. We conclude that mutant p53 may promote androgen-independent transition of CaP.

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## Figure legends

Fig. 1. Knock-down of wild-type p53 confers prostate cancer (CaP) cells resistance to DNA damage-induced apoptosis. (A) A representative clone of LNCaP-pTer-p53. LNCaP-pTer-p53 cells was uninduced (-tetracycline) or induced (+tetracycline) to express p53 siRNA for 24 hours. Cells were then treated with 300 nM Camptothecin (CPT) for O/N. p53 and actin were detected by anti-p53 and anti-actin antibodies, individually. (B) More LNCaP cells survive DNA damage when p53 is knocked down. LNCaP-pTer-p53 cells was uninduced (-tetracycline) or induced (+tetracycline) to express p53 siRNA for 24 hours. Cells were then treated with 300 nM CPT for 20h. On each day after CPT treatment, the cells that attached to the plates were counted. The survival on the day of CPT treatment was regarded as 100%. The survival ratio after CPT treatment was the product of the cell number on each day posttreatment divided by the cell number on Day 0. (C) Less LNCaP cells die after DNA damage when p53 is knocked down. LNCaP-pTer-p53 cells was uninduced (-tetracycline) or induced (+tetracycline) to express p53 siRNA for 24 hours. Cells were then treated with 300 nM CPT or 400  $\mu$ M 5-Fu for 20h. Three days after treatment, both live cells that attached to the plates and dead cells that floated in the medium were collected. Cells were stained with trypan blue dye. The ratio of dead cells was the product of dead cells divided by total cell number. (D) Long-term survival of LNCaP cells after DNA damage is increased when p53 is knocked down. LNCaP-pTer-p53 cells was uninduced (- tetracycline) or induced (+tetracycline) to express p53 siRNA for 24 hours. Cells were then treated with 0, 50, 100, or 200 nM Camptothecin (CPT) for 20h. 2 weeks after treatment, colonies were counted. The survival ratio was the product of the number of cell colonies at 50, 100, and 200 nM CPT groups divided by the number of colonies at the group without treatment.

Fig. 2. (A) A representative clone of DU-145-p53KD. (B) Less DU-145 cells survive DNA damage when mutant p53 is knocked down. (C) DU-145 cells are more sensitive to DNA damage-induced cell death. (D) Long-term survival of DU-145 cells is decreased when mutant p53 is knocked down.

Fig. 3. CaP cell death upon deprivation of androgen is independent of wild-type p53. LNCaP-pTer-p53 cells was uninduced (- tetracycline) or induced (+ tetracycline) to express p53 siRNA for 24 hours. The cells were then cultured in androgen-depleted medium for 3 days. The ratio of dead cells was determined as in Fig. 1C.

Fig. 4. Re-introduction of mutant p53 into wild-type p53 knock-down CaP cells further increases their resistance to DNA-damage induced apoptosis. (A) A representative clone of LNCaP-p53KD&p53(R175H). LNCaP and both uninduced and induced LNCaPp53KD&p53(R175H) cells were treated with 300 nM CPT for 20 hours. p53 and actin were detected as in Fig. 1A. (B) More LNCaP-p53KD cells survive DNA damage when p53(R175H) is inducibly expressed. (C) Less LNCaP-p53KD cells die after DNA damage when p53(R175H) is inducibly expressed.

Fig. 5. Re-introduction of mutant p53 into wild-type p53 knock-down CaP cells promotes their androgen-independent growth. LNCaP-p53KD&p53(R175H) cells were uninduced or induced to express p53(R175H) for 24h. The cells were then cultured in androgen-depleted

medium for 3 days. The ratio of dead cells was determined as in Fig. 1C.

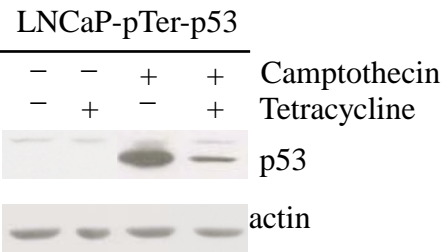
Fig. 6. p53 activation upon DNA damage is impaired by deprivation of androgen. LNCaP cell were cultured in medium containing regular fetal bovine serum or Charcoal-Dextrantreated fetal bovine serum for 24h. Cells were then treated with 300 nM CPT for 6h. Western blot was performed as in Fig. 1A.

Fig. 7. Identification of novel target genes of mutant p53. (A) MIA-PaCa-1-p53KD-4 and MIA-PaCa-1-p53KD-4 cells were un-induced (-) or induced (+) to express p53 siRNA for 72 hours. RNA was purified and Northern blot analysis was performed. (B) MIA-PaCa-1-p53KD-4 and MIA-PaCa-1-p53KD-4 cells were un-induced (-) or induced (+) to express p53 siRNA for 72 hours. Cell extracts were resolved and transferred to membrane. Western blot was performed by using anti-actin, anti-p53, anti-ECT2, and anti- $\beta$ -catenin.

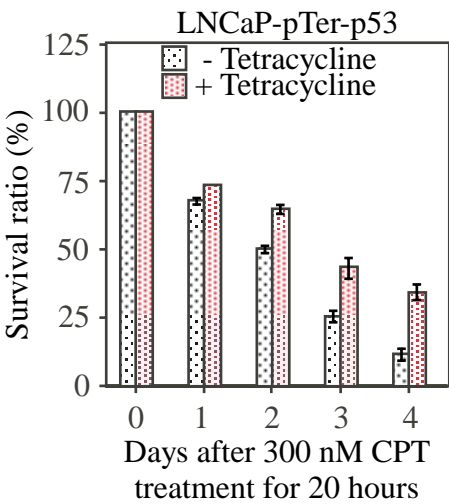
Fig. 8. NINJ1, a novel p53 target gene, potentiates p53 activities in inducing cell cycle arrest and apoptosis. (A) NINJ1 is up-regulated by p53, but not by p63 or p73. H1299 cells that were un-induced or induced to express p53, p63, or p73 for 24 hours. Total RNA was purified and Northern blot analysis was performed. (B) Up-regulation of NINJ1 by DNA damage is p53-dependent. Various cell lines were treated with 300 nM CPT for 24 hours. RNA was purified and Northern blot analysis was performed. (C and D) NINJ1 potentiates p53 activities in inducing cell cycle arrest and apoptosis. RKO (C) and MCF-7 (D) cells were un-induced or induced to express NINJ1-HA for 1 day. Then cells were treated with indicated concentration of Doxorubicin for 2 days. Cells were collected and FACS analysis was performed. The cells with sub-G1 DNA content were considered as apoptotic cells. (E) NINJ1 increases the p53 transcriptional activity. RKO cells were un-induced or induced to express NINJ1-HA for 24 hours. Cells were then treated with indicated drugs for 6 hours. Cell extracts were resolved and Western blot analysis was performed. (F) Localization of NINJ1 is unchanged by DNA damage. MCF-7 cells were induced to express NINJ1-HA for 24 hours. Cells were then treated with Doxorubicin for 16 hours. NINJ1-HA was detected with anti-HA monoclonal Ab. p53 was detected with anti-p53 polyclonal Ab. Nuclei were stained with DAPI.

Fig. 1

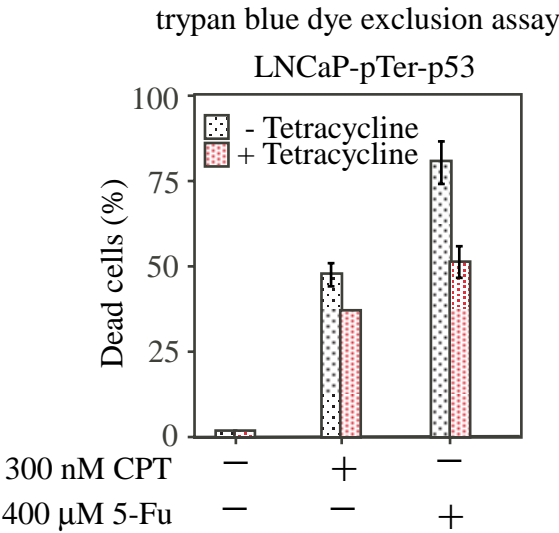
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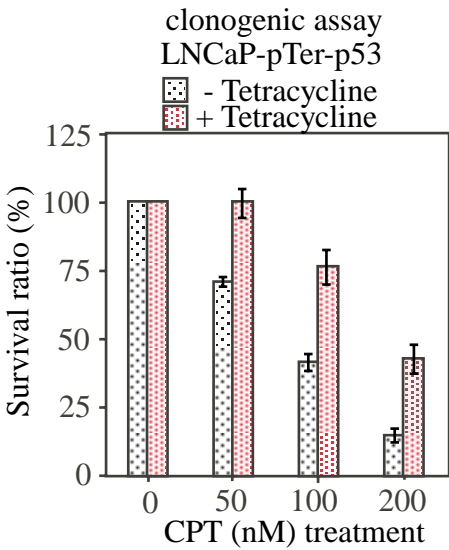
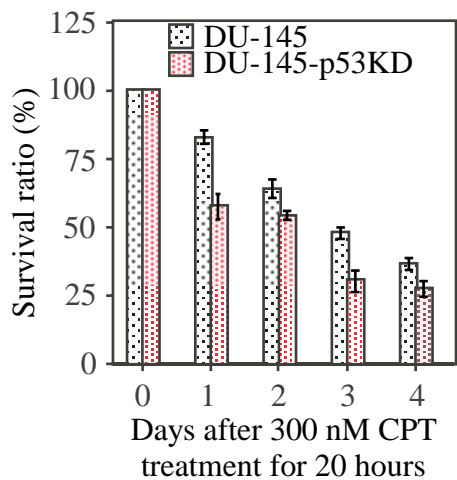


Fig. 2

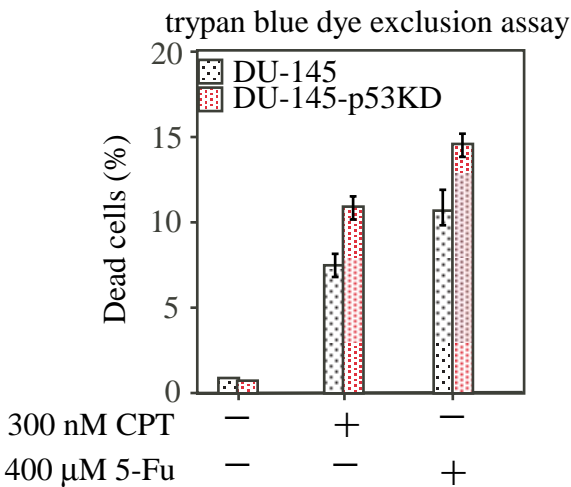
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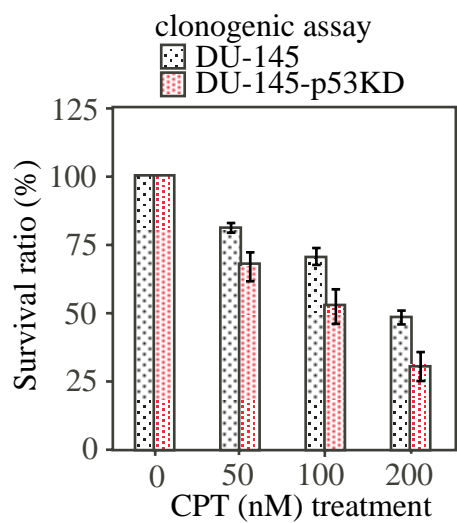


Fig. 3

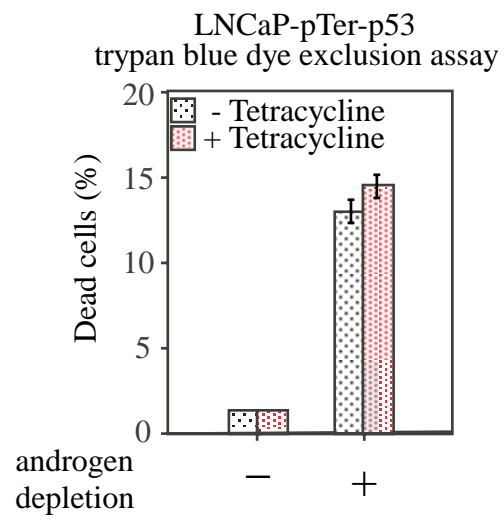
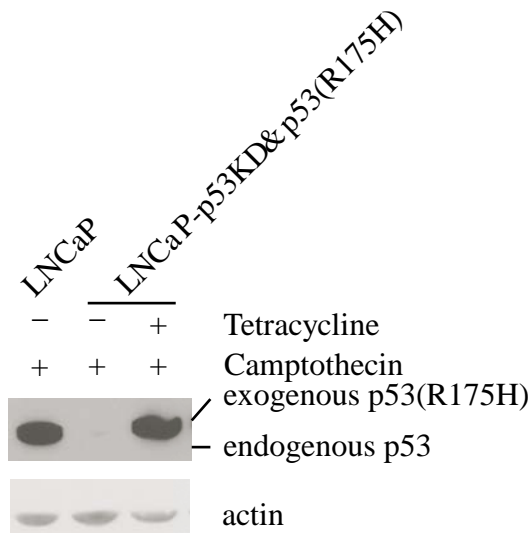


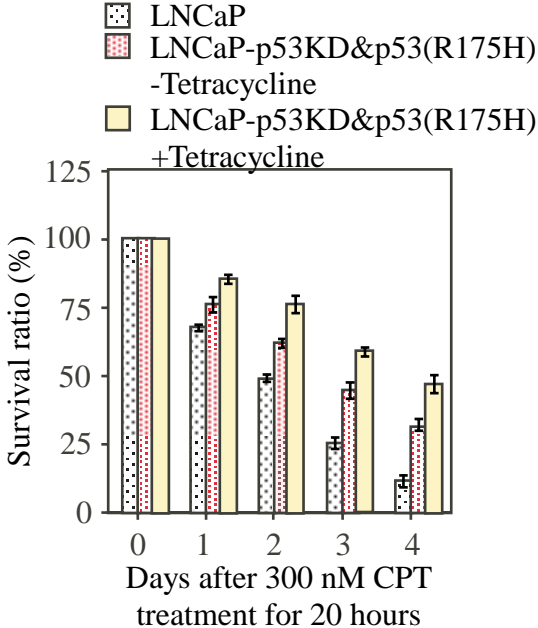


Fig. 4

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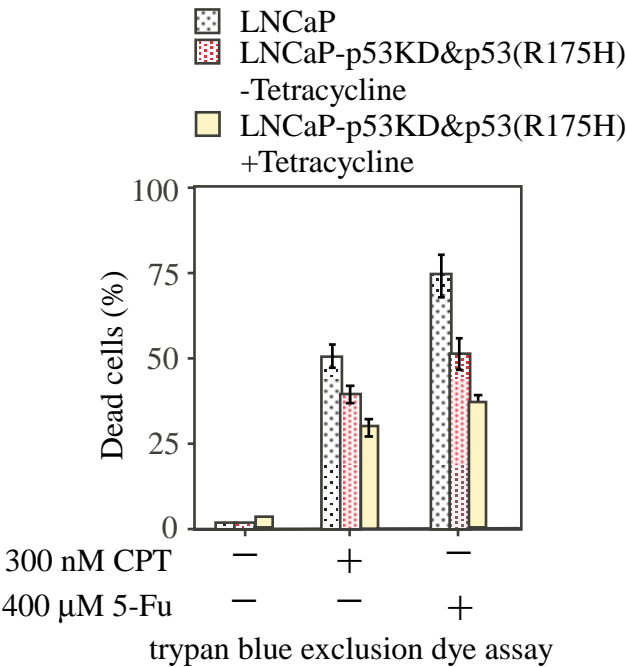


Fig. 5

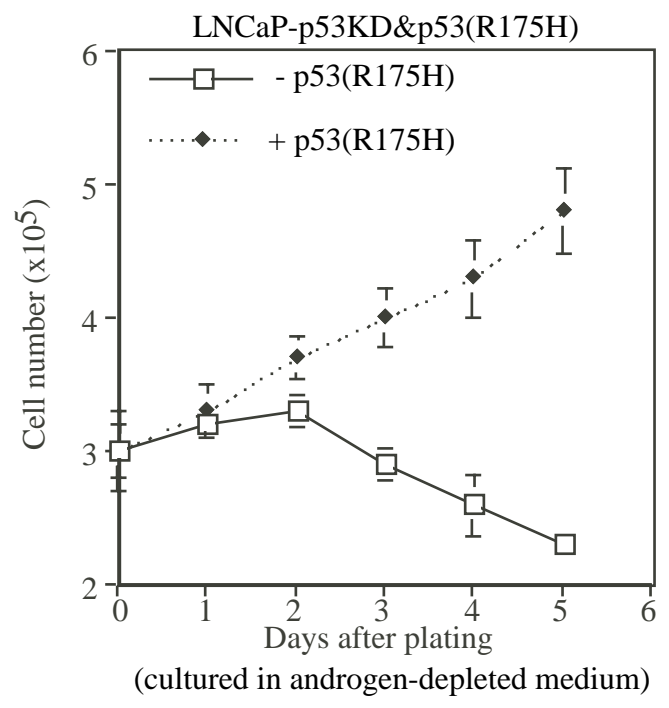


Fig. 6

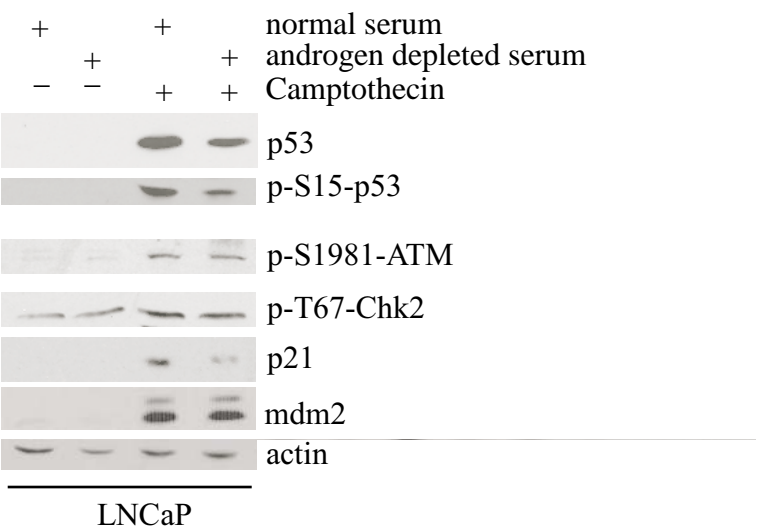
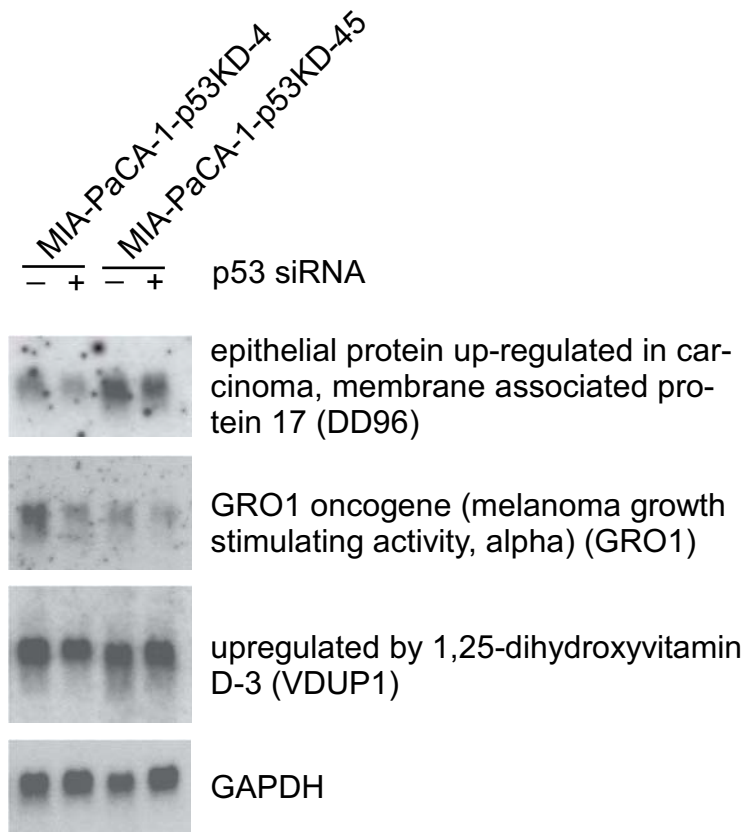


Fig. 7

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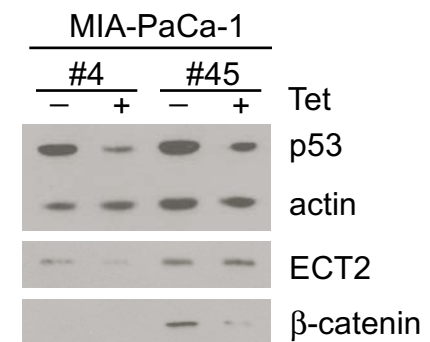
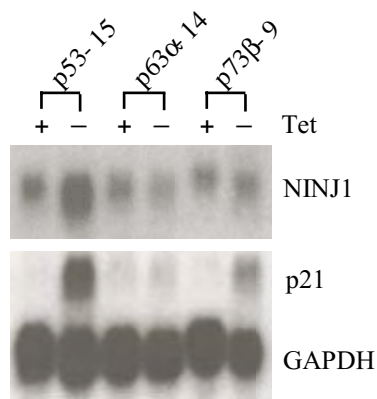
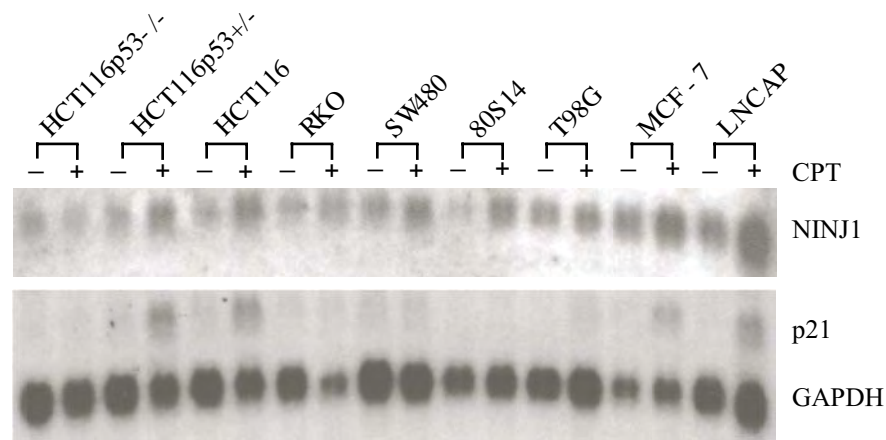


Fig. 8

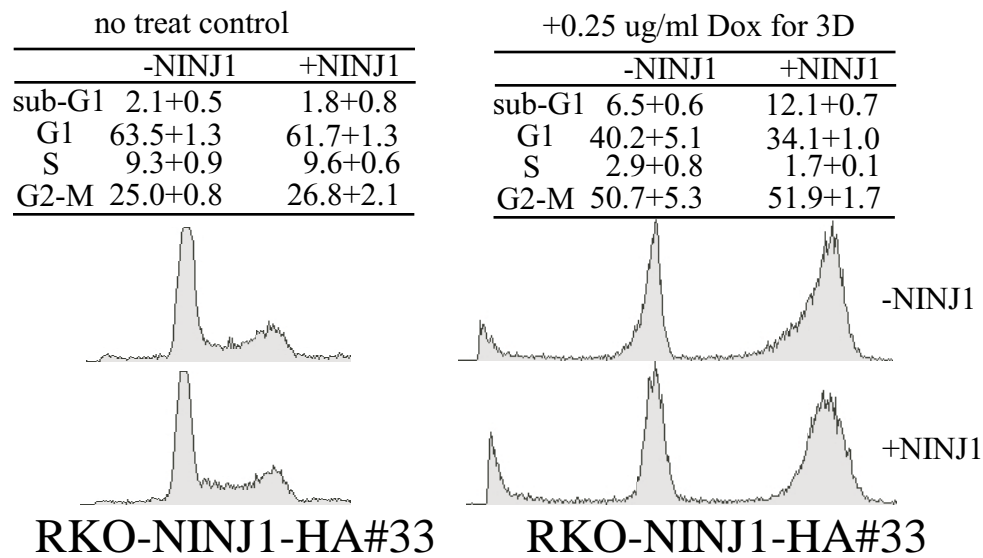
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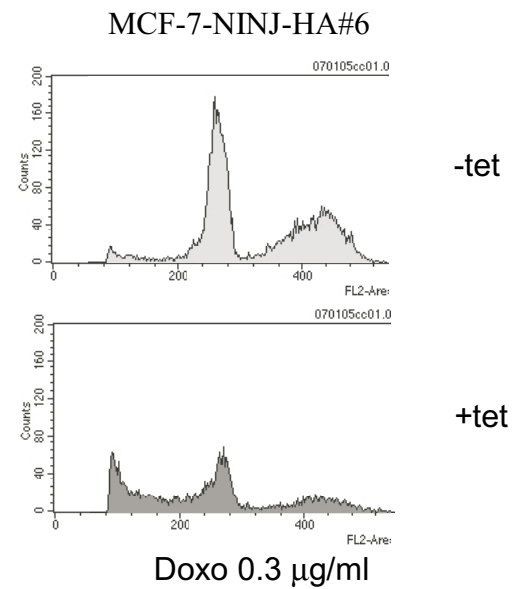
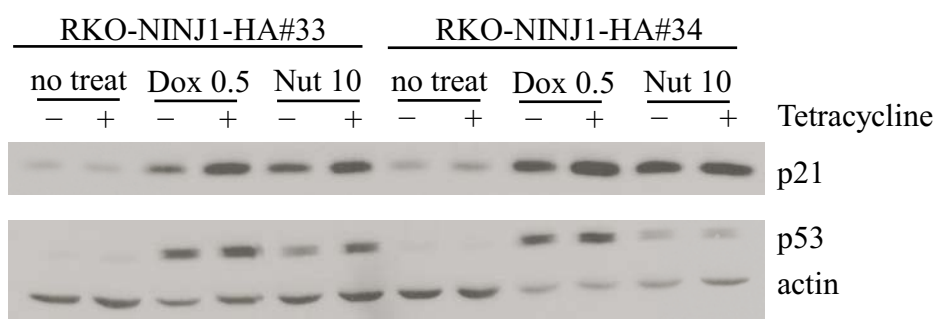


Fig. 8

E.



F.

